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Morphogenesis of the Middle Ear Ossicles and Spatial Relationships with the External and Inner Ears during the Embryonic Period

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ABSTRACT

We describe the three-dimensional morphogenesis of the middle ear ossicles (MEOs) according to Carnegie stage (CS) in human embryos. Seventeen samples including 33 MEOs from CS18 to 23 were selected from the Kyoto Collection. The primordia of the MEOs and related structures were histologically observed and three-dimensionally reconstructed from digital images. The timing of chondrogenesis was variable among structures. The stapes was recognizable as a vague condensation of the mesenchymal cells in all samples from CS18, whereas the malleus and incus were recognizable at CS19. Chondrogenesis of all MEOs was evident in all samples after CS21. The chondrocranium was recognizable in all samples by CS18, and the perichondrium border of the auricular cartilage and otic capsule was distinct in all samples at CS23. At CS19, the MEOs were positioned in the anterior to posterior direction, following the order malleus, incus, stapes, which adjusted gradually during development. The MEOs connected in all samples after CS22. The stapes was located close to the vestibular part of the inner ear, although the basal part was not differentiated into the “footplate” form, even at CS23. The handles of the malleus were close to the tubotympanic recess at CS23, but were distant from the external auditory meatus. Determining the timeline of the formation of MEOs and connection of the external and inner ears can be informative for understanding hearing loss caused by failure of this connection. These data may provide a useful standard for morphogenesis, and will contribute to distinguishing between normal and abnormal MEO development.

Key Words: Middle ear, human embryo, three-dimensional reconstruction, middle ear ossicles

INTRODUCTION

The middle ear contains three middle ear ossicles (MEOs), which are organized as a chain beginning from the malleus, followed by the incus, and ending in the stapes, that connect the tympanic membrane to the inner ear. The MEOs acquire an extremely elaborate and unique structure in order to effectively convey and amplify sounds from the tympanic membrane to the inner ear. The MEOs have a unique origin via condensation of the mesenchyme of the first two pharyngeal arches (Anson et al., 1961; Hanson et al., 1962; Rodríguez -Vázquez et al., 2006). However, the derivation of the auditory ossicles has not been completely resolved (Van De Water, 1980). In humans, the head of the malleus and the body and short process of the incus develop from the first pharyngeal arch. The handle of the malleus, the long process of the incus, and the head and legs of the stapes are believed to develop from the second pharyngeal arch (Anson et al., 1960). The base of the stapes appears to arise in the lateral wall of the otic capsule (Hanson et al. 1962). By contrast, other studies have shown that formation of the stapes is completely independent of the otic capsule (Louryan et al.,2003; Rodríguez -Vázquez et al., 2006). Moreover, it remains controversial as to whether Reichert's cartilage is the source of the stapes. Furthermore, recent studies have shown that the MEOs are neural crest derivatives (Ikeya et al., 1997; Mallo, 1997; Kanzler et al., 2000).

The morphogenesis of MEOs during the phase of mesenchymal condensation, and the appearance of each anlage have traditionally been evaluated using histological sections (Cauldwell & Anson, 1942; Altman, 1949; Richany et al., 1954; Anson et al., 1961; Hanson et al., 1962). However, most of these studies were conducted before the Carnegie staging system was established (O'Rahilly & Müller, 1987). Thus, it is difficult to directly compare and relate the classical findings with events that are ascribed to the currently employed Carnegie stages (CS). Overall, there is a general lack of data relating to the middle ear according to Carnegie staging of the human embryo (Van De Water & Ruben, 1976; O'Rahilly, 1983). It is important to establish the timeline of morphogenesis and the spatial relationship among each part of the auditory organs, including the MEOs, inner ear, external ear, and tubotympanic recess,

according to staged embryos, given that failure in any part of the chain of these structures could lead to a physiological deficiency (i.e., hearing loss). Recently, we analyzed the morphology of the inner and external ear in a stage-by-stage manner (Toyoda et al., 2015; Ozeki et al., 2016). Here, we provide details on the morphogenesis of the middle ear from CS18 to CS23, corresponding to chondrogenesis, using histological serial sections. The three-dimensional (3D) position and relationship of the MEOs with the external auditory meatus and inner ear were also determined. These results provide useful information on the morphological growth processes of the middle ear at each CS from CS18 to CS23.

MATERIALS AND METHODS

Human Embryo Specimens

Approximately 44,000 human embryos comprising the Kyoto Collection are stored at the Congenital Anomaly Research Center of Kyoto University (Nishimura et al., 1968; Shiota, 1991; Yamada et al., 2004). Most of these embryos were obtained when a pregnancy was terminated during the first trimester for socioeconomic reasons, under the Maternity Protection Law of Japan. Well-preserved human embryos with 10% formaldehyde fixation that were found to be externally morphologically normal were subjected to histological serial sectioning. For this study, a total of 17 human embryos with 33 middle ears between CS18 and CS23 and between 11.8 and 27.5 mm in crown-rump length (CRL) were selected from the Kyoto Collection.

Histological Observations

Histological serial sections (transverse sections, 10-μm thick) of whole embryos were scanned and stored as digital data using a film scanner (CanoScan 9000F, Canon, Tokyo, Japan) at 4800 dpi. The following primordia were histologically observed in the digital images: the malleus, incus, stapes, auricular cartilage, chondrocranium, membranous labyrinth, auditory capsule, and external auditory meatus.

Three-dimensional Reconstruction of the MEOs

Sequential two-dimensional images were trimmed digitally using ImageJ64 (ver.1.46, National Institutes of Health, Bethesda, MD, USA) and saved as a Microsoft Windows bitmap image (.bmp) at a resolution identical to that of the original digital file. The malleus, incus, stapes, inner ear membranous labyrinth, and external acoustic meatus were segmented, and 3D images were computationally reconstructed for examining the morphology in all samples using Amira software (ver. 5.5.0, Visage Imaging, Berlin, Germany). The optic cups and pituitary gland were used as an external anatomical reference point for standardization. Alignment was manually adjusted based on the external 3D view of the segmented primordia.

Each sample was categorized and confirmed after 3D reconstruction as follows: (-) impossible, not amply recognizable; (+/-) inaccurate, recognizable as a vague condensation of the mesenchymal cells; (+) possible, recognizable as increasing of the extracellular matrix; (++) precisely possible; distinct as the perichondrium border of the cartilage. The Ethics Committee of the Kyoto University Graduate School and Faculty of Medicine (E986) approved this study.

RESULTS

Chondrogenesis of Each Anlage of the MEO and Adjacent Structures

Chondrogenesis of the structures comprising the otic organs is initiated during CS18 to CS23 (Figs. 1–5). Interestingly, the specific timing of chondrogenesis was found to vary among structures. The stapes and auditory capsule were recognizable as a vague condensation of the mesenchymal cells in all samples as of CS18, whereas the malleus and incus were only recognizable as of CS19 (Table 1, Fig. 1). Chondrogenesis of all three MEOs was distinctly observed based on the perichondrium border of cartilage in all samples after CS21. Chondrogenesis of the chondrocranium preceded that of other structures; specifically, the occipital condyle was distinct based on the perichondrium border of cartilage in all samples after CS19 (Fig.1B-i). With respect to the auricular cartilage and auditory capsule, the timing of chondrogenesis followed

that of the other structures (Fig.1B-i, Fig. 2A,C); namely the perichondrium border of cartilage was only distinct in all samples at CS23 (Fig. 5B)

Morphogenesis of the MEO from CS18 to CS23

Each anlage was 3D-reconstructed for more detailed analysis (Table 2, Fig. 2). We found no obvious differences between the right side and the left side in the same embryo.

Malleus

The primordium of the malleus was not recognizable in any of the 8 organs at CS18, and was detected in 4 of 6 organs at CS19. The head of the malleus was detected in 2 of 4 organs at CS19, 2 of 4 organs at CS20, and in all 15 organs after CS21 (Fig. 3B,C). The handle of the malleus was not detected in any of the 8 organs reconstructed at CS19 and CS20, and was detected in 5 of 8 organs at CS21 and in all 7 organs after CS22 (Fig. 4C). The lateral and anterior processes were not detected, even at CS23. However, the os goniale, which is the premordium of the anterior process, was detected in histology (Fig. 5C). The tympanic membrane was also not formed, even at CS23. The malleus in all reconstructed organs was connected to Meckel's cartilage (shown as a broken line in Fig. 2C,3B,C, Fig. 4A, Fig. 5).

Incus and contiguation to adjacent MEOs

The primordium of the incus was not recognizable in any of the 8 organs at CS18, but was detected in 4 of 6 organs at CS19, and in all 19 organs after CS20 (Fig. 1, Fig. 2). The long process was not detected in any of the 6 organs at CS19, but was found in 2 of 4 organs at CS20, 5 of 8 organs at CS21, and in all 7 organs after CS22 (Fig. 2B,C, Fig. 3B, Fig. 4A,C, Fig. 5A). The short process was not detected in any of the 4 organs at CS19, but was detected in 2 of 4 organs at CS20, and in all 15 organs after CS21 (Fig. 3A, Fig. 4A). The short process reached the otic capsule.

Both the malleus and incus were recognizable in 4 of 6 organs at CS19. For the 4 organs in CS19 and CS20, the incus was contiguous from the surface to the malleus in 2 cases, but was not contiguous in the other 2 cases. The incus was

contiguous from the surface to the malleus in all 15 organs after CS21 (Fig. 3A, Fig. 4A, Fig. 5A). The incus was separate from the stapes in all 4 organs at CS19 and CS20, at which point both the stapes and incus could be clearly detected (Fig. 2B). The incus was contiguous from the surface to the stapes in 7 of the 8 organs at CS21, and in all 7 organs after CS22 (Fig. 4B, Fig. 5A).

Stapes

The primordium of the stapes was recognizable at an earlier stage than that of the incus or malleus, by one or two stages (Fig. 1A). The annular form of the stapes could already be observed in 2 of the 8 organs at CS18 and in all 25 organs after CS19 (Fig. 1B, Fig. 2B, Fig. 3A, Fig. 6). The stapes was closed to the otic capsule, although the cell density of the component was different and clearly delimited after CS19 (Fig. 1B-iii, Fig. 3A, Fig. 4A, Fig. 5A). The basal part of the stapes was not differentiated into the “footplate” form, as revealed by the 3D reconstruction, even at CS23. The stapes was not connected to the otic capsule in any the observed organs, and was instead closed to the mesenchymal lamina (Fig. 5A). The stapes was connected to the interhyale, which is the premordium of the tendon of the stapedius muscle, but not to Reichert’s cartilage, in all organs observed.

Spatial relationship between the MEOs, inner ear, and external auditory meatus

The three MEOs were positioned from the anterior to posterior direction as the malleus, incus, and stapes, in this order, at CS19 and CS20 in the lateral view (Fig. 7A). With respect to the connection between the inner ear and stapes, the stapes was closely located to the vestibular part of the inner ear, although the exact position of the oval windows could not be determined with the present method. The MEOs were compiled by lateral view, which adjusted gradually during development. All of the MEOs were connected after CS22 (Video 1).

The tubotympanic recess was differentiated from the pharynx, which ultimately becomes the auditory tubes and cavity of the middle ear later in development. It appeared flat by frontal view and a broad fissure extended to the middle and external ear anlage at CS18 (Fig. 1A). The tubotympanic recess became

narrower and lengthened after CS22 (Fig. 4C). The external auditory meatus was located ventrally and caudal to the MEO and tubotympanic recess at CS18, but was gradually positioned cranially, almost reaching the same level as the saccule, at CS23. The tubotympanic recess and handle of the malleus were closely located but were distant from the external auditory meatus at CS23 (Fig. 5B, Fig. 7B, Video 2).

DISCUSSION

The middle ear is composed of a cavity harboring a chain of MEOs that transmit and amplify the vibrations produced by airborne sound in the tympanic membrane into the inner ear, via the oval window. The schedule for the formation of these chains is an important topic, as abnormalities in the timing and connections between these structures can result in defects and hearing loss. The present study demonstrated that each MEO comes together forming a line, and the contiguation between the malleus and incus, and between the incus and stapes was already observed in all samples after CS22. By contrast, the connections between the inner ear and middle ear stapes, and between the malleus and external ear were still in progress during the present observation period (until CS23).

The malleus is composed of a main body with a series of processes. The handle of the malleus, which provides the physical link between the ossicle chain and the tympanic membrane, was observed after CS21. The 3D reconstruction showed that the handle was close to the tubotympanic recess, but distant from the external auditory meatus at CS23 (Fig. 7). The tympanic membrane itself was not obvious, even at CS23 (Fig. 5B,C). The os goniale, which becomes the anterior process (Rodriguez-Vazquez et al., 1991), was detected only in histology in the present study (Fig. 5C).

The stapes is composed of two essential components: the limbs and the base. The base provides the connection between the stapes and the inner ear. The 3D reconstruction revealed that the stapes was closely located to the vestibular part of the inner ear, although this part was not differentiated as a “footplate” at CS23. Previous studies indicated that the labyrinthine side of the

base of the stapes originates from the mesenchyme of the otic capsule (Cauldwell & Anson, 1942; Altman, 1949; Richany et al., 1954; Anson et al., 1961; Hanson et al., 1962). Masuda et al. (1977, 1978) observed that the primordium of the stapedial lamina forms in the 16-mm embryo, and the lamina is fully formed in the 35-mm embryo and fuses with the base of the stapedial annulus. However, Rodriguez-Vazquez (2005) described that the otic capsule is not involved in formation of the base of the stapes, and our observation supports this finding. Rodriguez-Vazquez (2009) also revealed the origin and development of the stapedius muscle, which is formed by two anlagen: one for the tendon, which is derived from the internal segment of the interhyale, and the other for the belly, which is located in the second pharyngeal arch medial to the facial nerve and near the interhyale but forms a completely independent anlage.

Most classical studies were conducted before the Carnegie staging system was established; thus, the embryo was previously analyzed according to the CRL and/or gestational weeks (Cauldwell & Anson, 1942; Altman, 1949; Richany et al., 1954; Anson et al., 1961; Hanson et al., 1962). Consequently, these older findings are difficult to reconcile with events ascribed to the currently employed CS system (Van De Water & Ruben, 1976; O'Rahilly, 1983). As mentioned above, although Rodriguez-Vázquez (2005, 2009) reported the formation of the stapes and related structures according to CS, all three MEOs were only reconstructed in one figure and only at CS20 (Table 3). Hanson et al. (1962) described the 3D structures of MEOs from 7- to 28-mm embryos, including the Carnegie collections. The CS of several of the embryos included in that study could be identified according to the Carnegie Series Number (O'Rahilly & Müller, 1987); however, in this study, the morphology was reconstructed at much earlier stages (CS16: 7.0, 8.0, and 9.6-mm CRL; and CS18: 11.7 mm CRL). In the present study, we could observe the primordia of the MEOs in the two-dimensional histological section as a very vague condensation of the mesenchymal cells at around CS16. We could further reconstruct the MEOs after CS18 in the present study. The 3D reconstruction was not accurate or was impossible to achieve for earlier stages because the border of the mesenchymal condensation was vague or not recognizable before CS18. The precise 3D reconstructions may be available 1 to 3 stages later than

the recognition of the mesenchymal condensation in histological sections (Table 3).

The present study reveals the morphological growth programs of the MEOs and the spatial relationship with the inner and external ears in staged human embryos from CS18 to CS23. These timelines are important to determine, as failure in any part of the chain of the structure could lead to physiological deficiency (hearing loss). These data are expected to provide a useful standard for morphogenesis, and should be helpful for distinguishing between normal and abnormal development.

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Figure legends

Abbreviations;
ac, auricular cartilage and auditory capsule; cc; chondrocranium; cd, cochlear duct; ct, chorda tympani nerve; ex, external auditory canal; f, facial nerve; fg, facial nerve ganglion;
I, incus; (Il, long process; Ib, body; Is, short process); ih, interhyale; M, malleus; (Ma, handle; Md, head); mc, Meckel's cartilage; ml, mesenchymal lamina; mp, meatus plaque;
oc, otic capsule; og, os goniale; ops, both the left and right optic cups were overlapped, ph, pharynx; pg, pituitary gland; rc, Reichert's cartilage anlage; S, stapes;(Sa, anterior limb; Sh, head; Sp, posterior limb); sac, saccule; sd, semicircular duct (sda, anterior semicircular duct; sdp, posterior semicircular duct); tm, tensor tympanic muscle; ts, tendon of stapedius muscle; tt, tubotympanic recess (auditory tube); ve, vestibule; *,stapes artery

Fig. 1 Histological features of the middle ear ossicles at CS18 (A) and CS19 (B).
(hematoxylin & eosin staining), scale bar indicates 200 μm.(CS18: ID 1250, CRL 13.9 mm; CS19: ID 2114,CRL 17.5 mm)
The stapes and auditory capsule were recognizable as a vague condensation of the mesenchymal cells in all samples as of CS18 **(A)**, whereas the malleus and incus were only recognizable as of CS19 **(B)**. The limbs of the stapes were differentiated (the stapedial artery passes between the stapedial limbs) **(B-ii)**.
ac, auricular cartilage and auditory capsule; cc; chondrocranium; ct, chorda tympani nerve; f, facial nerve; fg, facial nerve ganglion; I, incus; ic, internal carotid artery; M, malleus; oc, otic capsule; ph, pharynx; S, stapes; tt, tubotympanic recess (auditory tube); ve, vestibule; *,stapes artery;

Fig. 2 Histological features of the middle ear ossicles at CS20.
(hematoxylin & eosin staining), scale bar indicates 200 μm. (ID 12113, CRL 17.9 mm)
All three MEOs were distinctly observed. Chondrogenesis of the auricular

cartilage and auditory capsule followed that of the other structures **(A, B)**. The interhyale lies between the stapes and the second arch (Reichert's cartilage) **(C)**.

ac, auricular cartilage and auditory capsule; cc; chondrocranium; ct, chorda tympani nerve; ex, external auditory canal; f, facial nerve; Il, long process of incus; ih, interhyale; M, malleus;(Ma, handle of malleus); mc, Meckel's cartilage; oc, otic capsule; rc, Reichert's cartilage anlage; S, stapes; tm, tensor tympanic muscle; ve, vestibule; *,stapes artery;

Fig. 3 Histological features of the middle ear ossicles at CS21.

(hematoxylin & eosin staining), scale bar indicates 200 μm. (ID 1074, CRL 20.0 mm)

Chondrogenesis and differentiation of all three MEOs were distinctly observed based on the perichondrium border of the cartilage. Both the long and short processes, and the body of the incus were detected **(A, B)**. The short process reached the otic capsule **(A)**. The head of the malleus was detected. The malleus was connected to Meckel's cartilage **(B, C)**.

ct, chorda tympani nerve; f, facial nerve; I, incus; (Il, long process; Ib, body; Is, short process); M, malleus; (Ma, handle; Md, head); mc, Meckel's cartilage; oc, otic capsule; rc; Reichert's cartilage anlage; S, stapes; (Sa, anterior limb; Sp, posterior limb); tm, tensor tympanic muscle; ve, vestibule;

Fig. 4 Histological features of the middle ear ossicles at CS22.

(hematoxylin & eosin staining), scale bar indicates 200 μm. (ID 8002, CRL 20.8 mm)

The stapes was closed to the otic capsule, although the cell density of the components differed and could be clearly delimited **(A)**. The handle and head of the malleus were detected **(B, C)**. The tubotympanic recess became narrower and lengthened **(C)**.

cd, cochlear duct; ct, chorda tympani nerve; f, facial nerve; I, incus; (Il, long process; Ib, body; Is, short process); ih, interhyale; M, malleus; (Ma, handle; Md, head); mc, Meckel's cartilage; oc, otic capsule; rc, Reichert's cartilage anlage; S, stapes; (Sh, head); tm, tensor tympanic muscle; tt, tubotympanic recess

(auditory tube); ve, vestibule; *,stapes artery;

Fig. 5 Histological features of the middle ear ossicles at CS23.
(hematoxylin & eosin staining), scale bar indicates 200 μm. (ID 10273, CRL 27.5 mm)
The stapes was not connected to the otic capsule, and was instead closed to the mesenchymal lamina. The stapes was connected to the tendon of the stapedius muscle, but not to Reichert's cartilage **(A)**. The tubotympanic recess and handle of the malleus were closely located but were distant from the external auditory meatus **(B)**. The os goniale, which is the premordium of the anterior process, was detected in histology **(C)**.
ac, auricular cartilage and auditory capsule; cc; chondrocranium; cd, cochlear duct; ex, external auditory canal; f, facial nerve; I, incus; (Il, long process; Ib, body); ih, interhyale; M, malleus; (Ma, handle; Me, head); mc, Meckel's cartilage; ml, mesenchymal lamina; oc, otic capsule; og, os goniale; S, stapes; ts, tendon of stapedius muscle; tt, tubotympanic recess (auditory tube); ve, vestibule;

Fig. 6 Morphogenesis of the middle ear ossicles (MEOs) from CS18 to CS23. Right lateral view of the three MEOs (malleus, incus, and stapes).
I, incus; (Il, long process; Ib, body; Is, short process)
M, malleus; (Ma, handle; Md, head); mc, Meckel's cartilage
S, stapes; (Sa, anterior limb; Sh, head; Sp, posterior limb)

Fig. 7 Relative position between middle ear ossicles (MEOs) and internal ear, and external auditory meatus
A) The right oblique view and lateral views are shown. The border of the tubotympanic recess (tt) is indicated as a broken line. Pituitary gland and optic cups were used as anatomical landmarks.
ops, both the left and right optic cups were overlapped; pg, pituitary gland
B) Relationship among the bilateral ear organs, including the pharynx, tubotympanic recess, middle ear, and external auditory meatus at CS23.
cd, cochlear duct; I, incus; m, malleus and Meckel's cartilage; mp, meatus plaque; ph, pharynx; S, stapes; sac, saccule; sd, semicircular duct; (sda, anterior

semicircular duct; sdp, posterior semicircular duct)

Video 1. Relationship between the inner ear and middle ear at CS22.

Video 2. Relationship among the bilateral ear organs, including the pharynx, tubotympanic recess, inner ear, middle ear, Meckel's cartilage, and external auditory meatus at CS23.

Blue, internal ear membranous labyrinth; Green, pharynx and tubotympanic recess; Light purple, stapes; Purple, meatus plaque; Red, incus; Yellow, malleus and Meckel's cartilage

Table 1. Three-dimensional reconstruction of the auditory organs between Carnegie stages 18 and 23.

Structure	**Reconstruction**	**Carnegie stage**					
		18	**19**	**20**	**21**	**22**	**23**
Malleus	(++)			2	8	5	2
	(+)						
	(+/-)		4	2			
	(-)	8	2				
Incus	(++)			4	8	5	2
	(+)		3				
	(+/-)		1				
	(-)	8	2				
Stapes	(++)			2	8	5	2
	(+)		6	2			
	(+/-)	8					
	(-)						
Otic capsule	(++)						2
	(+)		6	4	8	5	
	(+/-)	8					
	(-)						
Auricular cartilage	(++)					3	2
	(+)						
	(+/-)		4	4	8	2	
	(-)	8	2				
chondrocranium	(++)		6	4	8	5	2
	(+)						
	(+/-)						
	(-)	8					
Total number of middle ear ossicles observed		8	**6**	4	8	5	2

(++): precisely possible, distinct as the perichondrium border of cartilage

(+): possible, recognizable as increasing of the extracellular matrix

(+/-): inaccurate, recognizable as vague condensation of the mesenchymal cells(-): impossible, not recognizable

Table 2. Findings of the three-dimensionally reconstructed middle ear ossicles between Carnegie stages 18 and 23

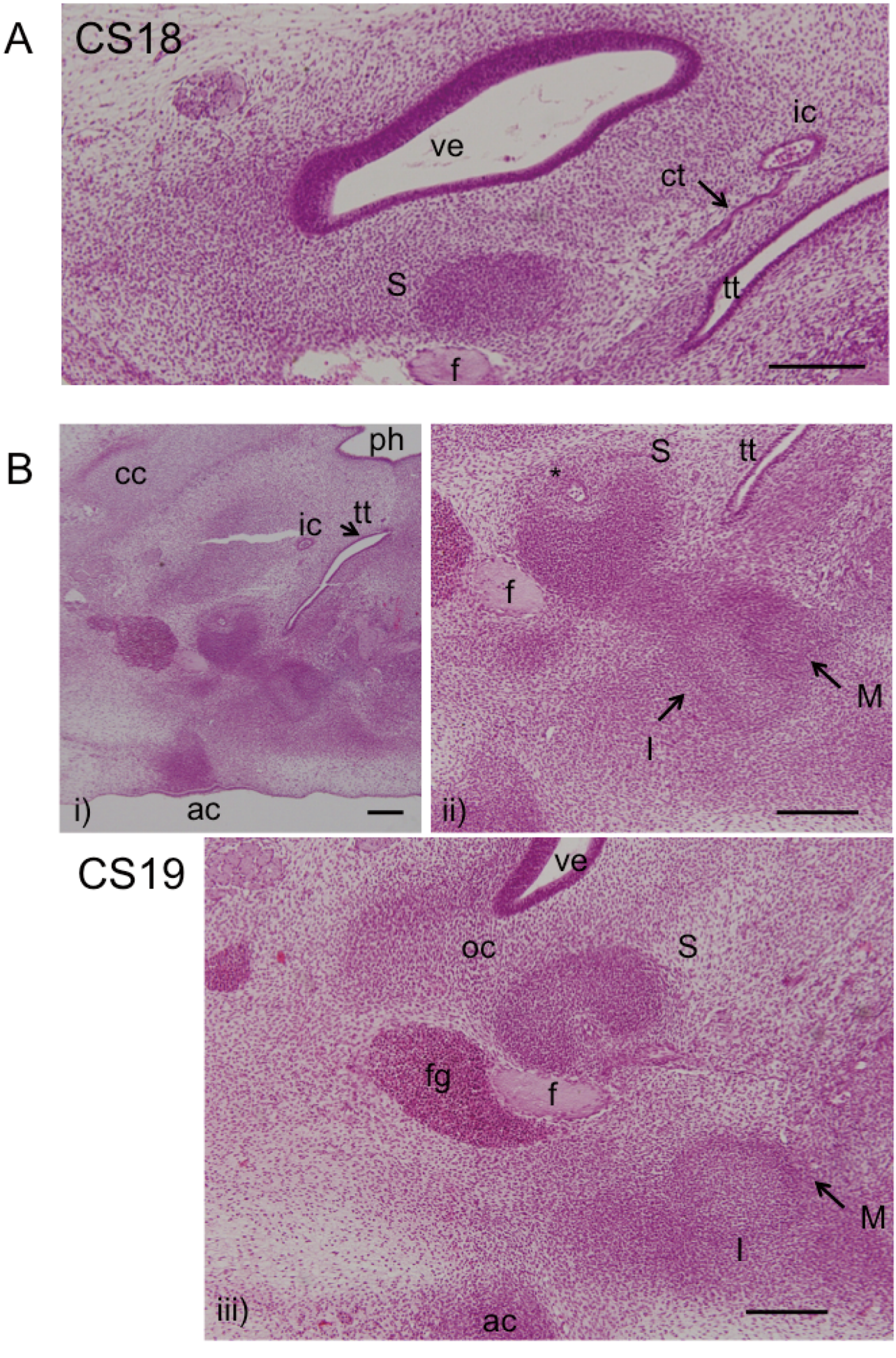
Parts of middle ear ossicles	Findings	Carnegie stage					
		18	19	20	21	22	23
Malleus: No. of reconstructible organs		0	4	4	8	5	2
Head and Neck	Recognizable		2	2	8	5	2
	Not recognizable		2	2			
Handle	Recognizable				5	5	2
	Not recognizable		4	4	3		
Anterior and lateral process	Not recognizable	0	4	4	8	5	2
Connection to Meckel's cartilage	connected		4	4	8	5	2
Incus: No. of reconstructible organs		0	4	4	8	5	2
Long process	Recognizable			2	5	5	2
	Not recognizable		4	2	3		
Short process	Recognizable			2	8	5	2
	Not recognizable		4	2			
Relation to malleus	Contiguous from the surface		2	2	8	5	2
	Not contiguous		2	2			
Relation to stapes	Contiguous from the surface				7	5	2
	Not contiguous		4	4	1		
Stapes: No. of reconstructible organs		8	6	4	8	5	2
Head	Recognizable			2	7	5	2
	Not recognizable	8	6	2	1		
Anterior and posterior limbs	Recognizable	2	5	4	8	5	2
	Not recognizable	6					
	Unknown		1				
Base	Not differentiated	8	6	4	8	5	2
Connection to Reichert's cartilage	Not connected	8	6	4	8	5	2

Total number of middle ear ossicles observed	8	6	4	8	5	2

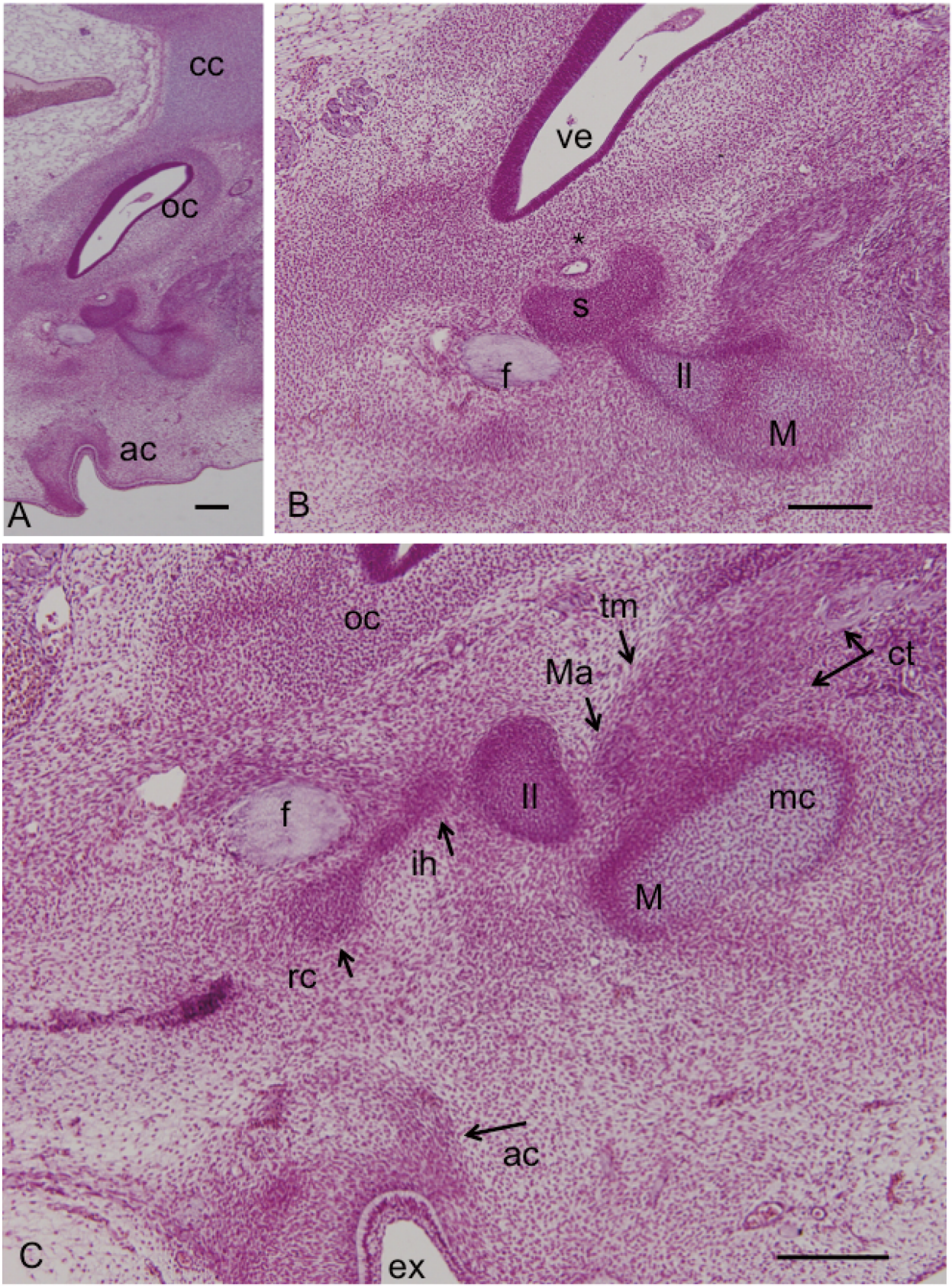
Table 3. Development of the middle ear ossicles and related structures.

		Carnegie Stages	
	Findings	Present study	Previous studies [ref][a]
Stapes	Delimitation of the parts of the stapedial anlage		17 [4]
	Stapes condensations (reconstruction inaccurate)	18	16–18 [1],18 [2],14 [4]
	Limbs differentiated (stapedial artery passes between the stapedial limbs)	19	17–18 [4]
	Interhyale lies between the stapes and the second arch (Reihert's cartilage)	20	18–20 [4]
	Stapes show evident chondrogenesis (precise reconstruction)	21	20 [4]
	Head differentiated	21	
	Disappearance of the stapedial artery	22	20 [4]
	Basal plate not connected to the otic capsule but close to the mesenchymal layer	23	22 [4]
	Basal part differentiated as a "footplate"	not until 23	22 [4] (kidney shape)
Incus	Incus condensations (reconstruction inaccurate)	19	17,18 [1],19 [2]
	Incus shows evident chondrogenesis (precise reconstruction)	20	20 [4]
	Long process differentiated	21	18 [4]
	Short process differentiated	21	20 [4]
	The short process reaches the otic vesicle	21	20 [4]
	Incus contiguous to the malleus	21	18 [4]
	Incus contiguous to the stapes	21	18 [4]
Malleus	Malleus condensations (reconstruction inaccurate)	19	17,18 [1],19 [2]
	Meckel's cartilage	19	18 [2]
	tensor tympanic muscle visible in reconstructions	20	20 [3]
	Malleus shows evident chondrogenesis (precise reconstruction)	21	20 [4]
	Head differentiated	21	20 [4]
	Handle differentiated	22	20 [4]
	Handles close to the tubotympanic recess, but distant from the external auditory meatus	23	
	Os gonieale observed	23	22 [5]
Other	Tubotympanic recess visible	<18	17 [3]
	Chorda tympani nerve visible	<18	17 [3]
	Otic capsule shows vague condensation (consisting of dense mesenchyme)	18	17 [2]
	Chondrocranium distinct (precise reconstruction)	19	
	Otic capsule distinct (precise reconstruction)	23	23 [2]
	Auricular cartilage distinct (precise reconstruction)	23	

[a][1] Hanson et al., 1962; [2] O'Rahilly and Müller, 1984; [3] Blechschmidt, 1963; [4] Rodriguez-Vazquez, 2005; [5] Rodriguez-Vazquez, 1991



CS20



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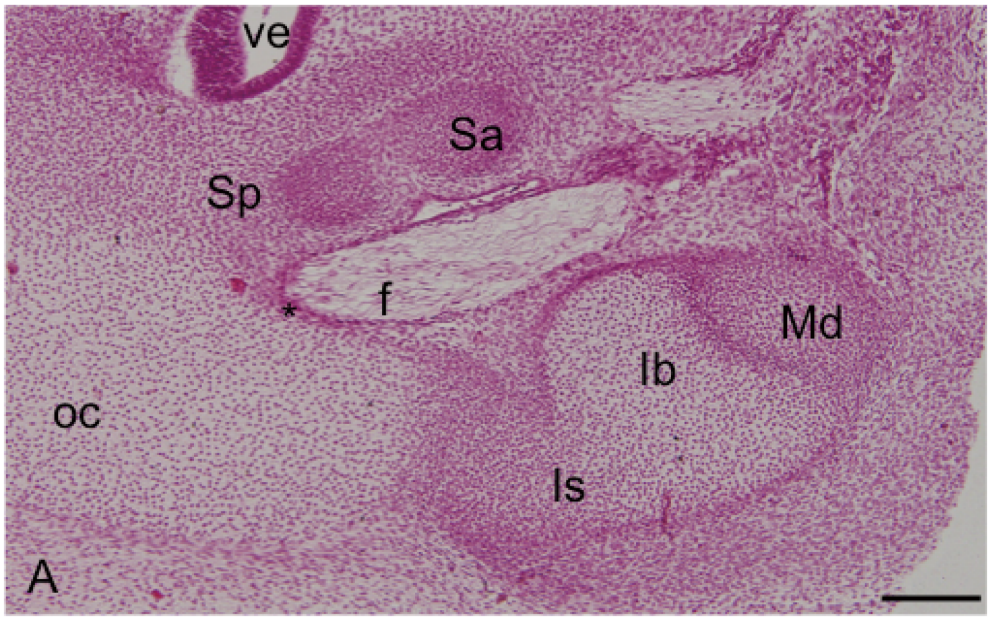

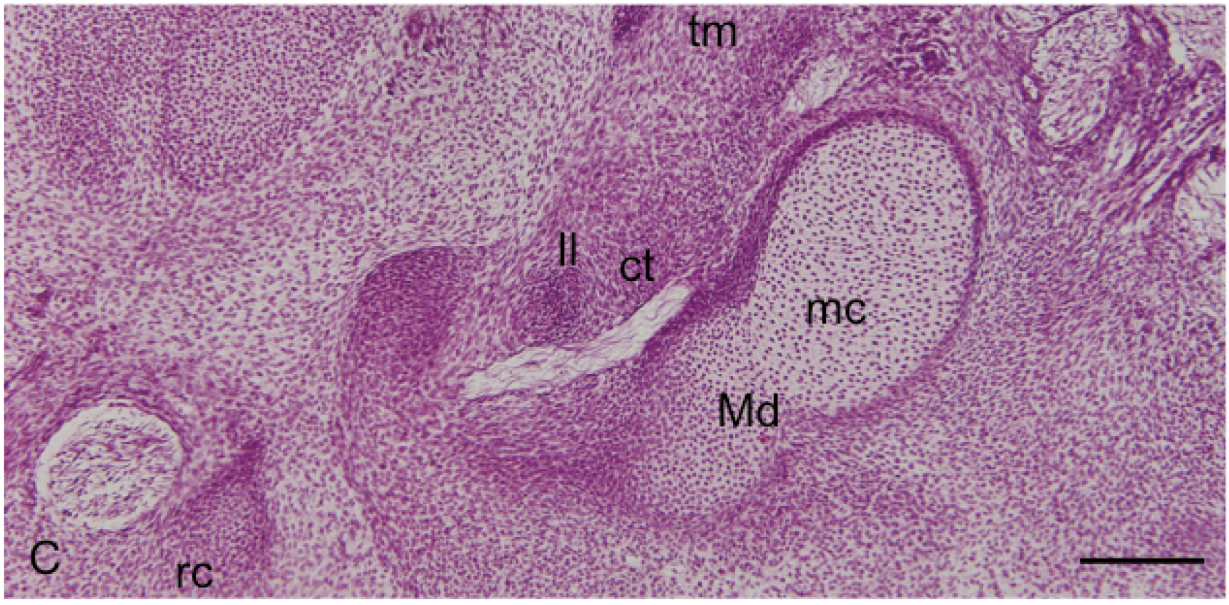

CS22